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<b>(54) Title:</b> PRRSV ANTIGENIC SITES IDENTIFYING PEPTIDE SEQUENCES OF PRRS VIRUS FOR USE IN VACCINES OR DIAGNOSTIC ASSAYS			
<b>(57) Abstract</b> <p>The invention provides antigenic sites of PRRSV isolates. The antigenic sites are neutralizing, conserved, non-conserved and conformational, can elicit antibodies and are found on protein GP<sub>4</sub> and N encoded by ORF4 and ORF7 of PRRSV. The peptide sequences identified by the sites can be incorporated in vaccines directed against PRRS and in diagnostic tests for PRRS. Also, discriminating tests can be developed that can be used next to marker vaccines in programs designed to eradicate PRRS from pig herds.</p>			

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Title: PRRSV antigenic sites identifying peptide sequences of PRRS virus for use in vaccines or diagnostic assays

The invention relates to the causative agent of Mystery Swine Disease, the PRRS virus, to peptide sequences identified in the PRRS virus, and to incorporating these sequences in vaccines and diagnostic tests.

5 PRRS virus (PRRSV) is the causative agent of a pig disease, currently called porcine reproductive and respiratory syndrome (PRRS). The virus is the causative agent of a pig disease, seen since approximately 1987 in the US and since 1990 in Europe, known initially under various names  
10 such as Mystery Swine Disease, Swine Infertility and Respiratory Syndrome, and many more. The virus itself was also given many names, among which Lelystad virus (LV), SIRS virus, and many more, but is now mostly designated porcine reproductive and respiratory syndrome virus (PRRSV). It  
15 causes abortions and respiratory distress in pigs and was first isolated in Europe in 1991 (EP patent 587780, US patent 5,620,691) and subsequently in the US and many other countries throughout the world. PRRSV is a small enveloped virus containing a positive strand RNA genome. PRRSV  
20 preferentially grows in macrophages. In addition to macrophages, PRRSV can grow in cell line CL2621 and other cell lines cloned from the monkey kidney cell line MA-104 (Benfield et al., J. Vet. Diagn. Invest. 4; 127-133, 1992). The genome of PRRSV, a polyadenylated RNA of approximately 15  
25 kb was sequenced in 1993 (Meulenberg et al., Virology 192; 62-74, 1993). The nucleotide sequence, genome organization and replication strategy indicated that PRRSV is related to a group of small enveloped positive-strand RNA viruses, designated Arteriviruses. This group includes lactate  
30 dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). These viruses have a similar genome organization, replication

strategy, morphology, and amino acid sequence of viral proteins. Arteriviruses contain a genome of 12.5 to 15 kb and synthesize a 3' nested set of six subgenomic RNAs during replication. These subgenomic RNAs contain a leader sequence which is derived from the 5' end of the viral genome. ORFs 1a and 1b comprise approximately two thirds of the viral genome and encode the RNA dependent RNA polymerase. Six smaller ORFs, ORFs 2 to 7, are located at the 3' end of the viral genome. ORFs 2 to 6 likely encode envelope proteins whereas ORF7 encodes the nucleocapsid protein (Meulenberg et al, Virology 206; 155-163, 1995).

PRRSV is the first Arterivirus for which it has been demonstrated that all six proteins encoded by ORFs 2 to 7 are associated with the virion. The 15-kDa N protein (encoded by ORF7) and the 18-kDa integral membrane protein M (ORF6) are not N-glycosylated, whereas the 29- to 30-kDa GP<sub>2</sub> protein (ORF2), the 45- to 50-kDa protein GP<sub>3</sub> protein (ORF3), the 31- to 35-kDa GP<sub>4</sub> protein (ORF4), and the 25-kDa protein GP<sub>5</sub> (ORF5) are. These proteins have also been detected in extracellular virus and lysates of cells infected with a North American isolate of PRRSV, ATCC-VR2332, and other isolates of PRRSV (other isolates of PRRSV are for example CNCM I-1140, ECACC V93070108, CNCM I-1387, CNCM I-1388, ATCC-VR2402, ATCC-VR2429, ATCC-VR2430, ATCC-VR2431, ATCC-VR2475, ATCC-VR2385, but many others are known).

We earlier described the isolation and characterization of a panel of PRRSV-specific MAbS that were specific for GP<sub>3</sub>, GP<sub>4</sub>, M and N (van Nieuwstadt et al., J. Virol. 70, 4767-4772, 1996). Interestingly, MAbS directed against GP<sub>4</sub> were neutralizing, suggesting that at least part of the protein is exposed at the virion surface. Furthermore, most of the MAbS directed against N reacted with all PRRSV isolates tested.

PRRS in it self is a problem of major concern for the swine industry in most parts of the world. Introduction of PRRSV in

pig herds will cause severe economic losses. Diagnostic testing against PRRS is widely practiced by many veterinarians and laboratories. Most diagnostic tests, such as IPMA, IFT, IFA, ELISA, each comprising suitable means of detection such as conjugated enzymes or fluorochromes, and other substrates, use interactions between antigen derived from PRRSV and antibodies directed against PRRSV to measure the presence of either PRRSV antigen or antibodies directed against PRRSV in a biological sample, such as blood, serum, tissue, tissue fluids, lavage fluids, urine, faeces, that is sampled from the animal (such as a pig) to be tested. The antigen and/or antibodies used in these diagnostic tests, or diagnostic kits or assays, for PRRS diagnosis are only defined by their origin from, or by their reactivity with PRRSV. In principle this suffices for screening assays where a high specificity or sensitivity is not explicitly required. However, the ever continuing spread of PRRS has caused great concern among the pig industry, to the extent that it is deemed needed to eradicate PRRS from whole herds, or even from complete areas, regions, or countries where pigs are raised. A clear example of this need is the proposed eradication program relating to PRRS in Denmark. If one decides to completely eradicate PRRS then diagnostic tests are needed that exhibit higher specificity or sensitivity than the tests used today.

Vaccination against PRRS is also widely practiced. Several examples are known of modified live vaccines that are used, and also killed vaccines are known. However, a problem with live vaccines in general, and thus also with live PRRS vaccines, exists in that these vaccines have a tendency to spread to non-vaccinated pigs, thereby spreading instead of reducing detectable infection in pig herds, and thus being counter productive to complete eradication. If a line marker vaccine were used that could serologically be differentiated from the wild type virus, then this problem would be greatly reduced. Added disadvantages are that live vaccines sometimes cause anaphylactic reactions in the vaccinated pigs, because

of undefined antigenic components. Although killed vaccines in general are reported to induce protection in the vaccinated pig, and have the additional advantage that they do not spread from pig to pig, a disadvantage of killed vaccines is that it may be hard to accrue sufficient antigenic mass in one dose of a vaccine to elicit a measurable and protective immune response. Especially killed vaccines that can induce measurable neutralizing antibody titers in pigs would be beneficial to have since measuring these neutralizing antibodies in vaccinated pig populations would help generate understanding about the level of protection obtained by vaccination in the pig herd. In addition, if one succeeds in assembling the necessary antigenic mass, this also means that more and other undefined antigenic mass is also present in the vaccine, which can also give rise to the anaphylactic reactions as described above. In this sense it would be beneficial to know which specific site on PRRSV is involved in neutralization, leading to the design of better suited vaccines, incorporating the important peptide sequences needed for eliciting neutralizing antibodies. An advantage of the currently used vaccines originating from PRRSV isolates isolated in the US is that such vaccines, albeit fully protective against and immunologically cross-reactive with European isolates of PRRSV, contain, as yet undefined, epitopes or antigenic sites by which they can be discerned from European isolates of PRRSV. Reciprocally, live vaccines originating from PRRSV isolates isolated in Europe, albeit fully protective against and immunologically cross-reactive with US isolates of PRRSV, contain similar as yet undefined epitopes or antigenic sites by which they can be discerned from US isolates of PRRSV.

If serological tests would be available which could discriminate (based on the small epitopic differences between PRRSV isolates) between pigs that are either vaccinated with a US derived vaccine or infected with a European wild type of PRRSV (being vaccinated or not), or which could discriminate pigs that are either vaccinated with a European derived

vaccine or infected with an US wild type of PRRSV (being vaccinated or no), than marker vaccines and corresponding diagnostic tests (incorporating said discerning epitopes or antigenic sites) could be developed which could be used with  
5 large confidence in eradication programs for PRRS. For example, in Denmark it would than be possible to vaccinate with a US derived vaccine and measure the set of antibodies in the Danish pigs which are solely directed against unique epitopes on European wild types of PRRSV and not cross-  
10 reactive with US strains. This would enable the unequivocal detection and subsequent removal of wild type infected pigs from Danish herds. Currently, such a discrimination is not possible due to the overall broad immunological cross-reactivity between PRRSV isolates. It goes without saying  
15 that such combined vaccination-testing programs will be the basis for eradication of PRRS, and can also be used in other countries, if needed with distinct PRRSV antigenic sites being used in vaccine and/or diagnostic test.

The invention now provides antigenic sites comprising  
20 peptide sequences of PRRSV which allow the improvement of vaccines, be it killed or attenuated vaccines or vaccines derived via recombinant DNA technology, and antigenic sites which allow the improvement of diagnostic methods, tests and kits and the production of new diagnostic methods, tests and  
25 kits. Artificial changes or amino acid residue substitutions that maintain the antigenicity (as for example defined by the reactivity with polyclonal sera or MAbs) and thus functionality of the antigenic site can easily be derived from sequences known to constitute an antigenic site of a  
30 specific isolate by a person with ordinary skills in the art of peptide design and synthesis. For example, certain amino acid residues can conventionally be replaced by others of comparable nature, e.g. a basic residue by another basic residue, an acid by an acid, a bulky by a bulky, a  
35 hydrophobic or hydrophilic by another hydrophobic or hydrophilic residue, and so on. Also, other, less conventional but more specific changes are also possible that

maintain or even improve the antigenicity of the selected sequence. Such changes can for example be made by PEPSCAN based amino acid substitutions or replacement mapping techniques (van Amerongen et. al., Peptide Research (1992) 5, 269-274. In short, amino acid residues within the antigenic sites provided by the invention can e.g. be replaced conventionally or under guidance of replacement mapping, whereby the resulting peptide sequences are functionally equivalent to the antigenic site. The replacing amino acids can be either L- or D- amino acid residues. In addition, the peptide sequences provided by the invention are rendered even more immunogenic by conjugating them to adjuvants (such as KLH) known in the art. Additionally, the peptides are rendered even more immunogenic by making peptides with one (such as tandem peptides) or more repeated sequences or by polymerization or circularization.

Although it has been shown before that the N protein is immunogenic (Meulenberg (1995), J. Clin. Diagn. Lab. Immunol. 2, 652-656, GB 2 289 279 A) and that conserved and nonconserved regions between the N protein of European strains (LV) and US strains (VR2332) exist (WO 96/04010), we demonstrate here for the first time which conserved and nonconserved regions are antigenic and which can be used individually or in combinations as antigens for immunization or diagnostic assays. Furthermore it is identified here that the antigenic regions in the N protein consist both of linear and conformation dependent epitopes.

The GP4 protein is the first structural protein of PRRSV for which is shown that it elicits antibodies that can neutralize the virus. A specific region of approximately 40 amino acids was identified and defined that should be exposed at the virion surface as a target for neutralizing antibodies, which then prevent the virus to infect the cells. This is an exciting new finding since it is generally assumed that the GP5 protein, the major structural of PRRSV, is the most important candidate involved in the attachment of the host cell.



The invention provides a major antigenic site, a neutralization site on GP<sub>1</sub> of PRRSV. The invention provides the localization of a major neutralization site important for the design of effective marker vaccines that comprise amino acid core sequences and amino acid sequences flanking the core sequences of PRRSV isolates which sequences comprise the neutralization site on the ORF4 protein of PRRSV. By incorporating the relevant neutralization site sequences in the various types of vaccines, it is possible to specifically induce neutralizing antibodies in the vaccinated pig. Killed vaccines comprising the neutralization site provided by the invention are made to induce measurable neutralizing antibodies. Especially sequences located at positions in the ORF 4 encoded protein of PRRSV corresponding to those found at about amino acid 40 to 79 as found in PRRSV isolate I-1102 comprise the neutralization site. Furthermore, selected peptide sequences are made even more immunogenic by mixing the peptides with adjuvants or other carriers known in the art. The thus obtained peptide compositions are used as a vaccine. However, also the selected peptide sequences comprising the neutralization site are incorporated in vaccine vector systems, being either distinct recombinant vectors derived of heterologous viruses or bacteria, but the selected peptide sequences are also selectively incorporated in PRRSV vector viruses or vaccines derived thereof.

In a further aspect of the invention, amino acid sequences located at positions corresponding from about 52 to 75 more specifically constitute a broadly reactive neutralization site. Other embodiments of the neutralization site provided by the invention can be found among the various PRRSV isolates known or to be found (see for instance the experimental part of this description). It is easy for any person working in the field of molecular biology to compare the sequences comprising the neutralization site provided by the invention with the amino acid sequence of the ORF 4 encoded protein of yet another PRRSV isolate.

The invention also provides peptide sequences of PRRSV which improve diagnostic tests, be it antigen or antibody detection tests. The invention provides various groups of antigenic sites which are used alone or in combination in diagnostic tests. In this way diagnostic tests are provided by the invention that serve the various needs that exists in the field with regard to diagnosis and differential diagnosis. Antigen-antibody interactions always entail cross-reactive epitope-paratope interactions of amino acid sequences that are from 5 to 15 amino acid sequences long. Thus amino acid sequences of 5 to 15 amino acids long and partly or completely overlapping with the core sequences of the antigenic sites of invention are provided by the invention for incorporation in diagnostic tests. These peptide sequences are used to select or design antigen or antigenic substance containing the sequences in the test to be used. Alternatively, and provided by the invention, are synthetic antibodies reactive with the antigenic sites provided by the invention. These sites or related sequences react with synthetic antibody obtained from systems such as phage display libraries or clonal selection of (heavy chain) antibodies that constitute antibody-like molecules which can easily be expressed in heterologous expression systems.

One group provided by the invention comprises the peptide sequence corresponding to said neutralization site, as already explained above. Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect neutralizing antibodies in the pig. Another group provided by the invention comprises a conserved antigenic site on protein N. Within the conserved antigenic site the invention provides a core sequence VNQLCQLLGA or VNQLCQMLGK. Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect those antibodies in pigs that specifically react with most PRRSV isolates. Also, diagnostic tests are provided that use antibodies directed against the conserved site to detect the

PRRSV antigen, thereby allowing the test to detect PRRSV isolates, irrespective of their origin.

Another group provided by the invention comprises a non-conserved differentiating antigenic site on protein N.

5 Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect those antibodies in pigs that specifically react with distinct PRRSV isolates, whereby for example vaccinated pigs can be discriminated from pigs infected with wild type PRRSV. Also,  
10 diagnostic tests are provided that use antibodies directed against the non-conserved site to detect the PRRSV antigen, thereby allowing the test to discern different PRRSV isolates. Within one such a non-conserved site the invention provides a core sequence PRGGQAKKKK or PRGGQAKRKK or  
15 PRGGQAKKRK or GPGKKNKKKK or GPGKKNKKKT or GPGKKNRKKK or GPGKKFKKKK or GPGKKIKKKK or GPGQINKKIN. Within another non-conserved site the invention provides a core sequence MAGKNQSQKK or MPNNNGKQTE or MPNNNGKQPK or MPNNNGKQQK or MPNNNGKQQN or MPNNNGKQQK or MPNNNGRQQK. Also, artificial  
20 changes that maintain the antigenicity and thus functionality of the above core sequences in the GP4 or N protein can easily be introduced by anyone skilled in the art of peptide design and synthesis, as described above.

The invention also provides a group comprising  
25 conformational epitopes (which vary greatly among the various isolates) which can be found at positions corresponding to those found in isolate I-1102 from amino acid position 51 to about 68 (in isolate I-1102 core sequence PKPHFPLAAEDDIRHHL) or from 79 to about 90 (in isolate I-1102 core sequence  
30 SIQTAFNQGAGT) or from 111 to 124 (in isolate I-1102 core sequence HTVRLIRVTSTSAS) on protein N. The conserved and non-conserved and differentiating and conformational sites in the N protein, which sites are provided by the invention, provide diagnostic tests that unequivocally diagnose PRRSV  
35 infections. Tests are made that avoid employing non-conserved sites thereby avoiding false-negative results. In addition, the various non-conserved sites are used in the development

of differentiating tests that can e.g. discriminate vaccinated pigs from pigs infected with wild type isolates of PRRSV. Again, as said it is easy for any person working in the field of molecular biology to align the sequences comprising the conserved or non-conserved or conformational epitope sites with amino acid sequences of the ORF 7 encoded protein of yet another PRRSV isolate. The sites provided by the invention are used in new pairs of vaccine-discriminating diagnostic tests for use in eradication programs of PRRS.

10

## Experimental part

### Materials and methods

#### Cells and viruses

15       The Ter Huurne strain (CNCM I-1102) of PRRSV was isolated in 1991 (Wensvoort et al., 1991). The US ATCC-VR2332 strain was isolated by Benfield et al. (1992). Strain NL1 (Netherlands, 1991) was isolated in our lab, Strain NY2 (England, 1991) was kindly provided by T. Drew, strain DEN  
20       (Denmark, 1992) was kindly provided by A. Botner, strain LUX (Luxemburg, 1992) was kindly provided by Losch, SPA1 and SPA2 (Spain, 1992) were kindly provided by Shokouhi and Espuna, respectively, and strain FRA (France, 1992) was kindly provided by Y. Leforban.

25       PRRSV and VR2332 were grown on CL2621 cells as described previously (van Nieuwstadt et al., 1996). The seven different European isolates were grown in porcine alveolar macrophages. Macrophages were maintained as described before (Wensvoort et al, 1991). BHK-21 cells were maintained in Dulbecco's  
30       Minimal Essential Medium supplemented with 5% fetal bovine serum and antibiotics. For transfection experiments, BHK-21 cells were grown in Glasgow Minimal Essential Medium (GIBCO-BRL/Life Technologies Ltd).

#### Antisera.

Porcine anti-PRRSV serum 21 and rabbit anti-peptide sera 698 and 700 were used in previous experiments. Serum 700 is directed against amino acids 106 to 122 (CLFYASEMSEKGFKVIF) encoded by ORF4 of PRRSV and was obtained from a rabbit. The production and characterization of MAbs have been described (van Nieuwstadt et al, 1996). The hybridomas were derived from five consecutive fusion experiments and directed against ORF 4 protein (MAb 121.4, 122.1, 122.12, 122.20, 122.29, 122.30, 122.59, 122.66, 122.68, 122.70, 122.71, 126.1, 126.7, 130.7, 138.28) or ORF 7 protein (MAb 122.17, 125.1, 126.9, 126.15, 130.2, 130.4, 131.7, 138.22, WBE1, WBE4, WBE5, WBE6, SDOW17) MAbs WBE were graciously provided by Dr. Drew, Weybridge, UK; MAb SDOW17 was graciously provided by Dr. Benfield, South Dakota, US.

#### Plasmid constructions

Two oligonucleotides located upstream (PRRSV13) and downstream (PRRSV14) of ORF4 have been used earlier to amplify and clone ORF4 of isolate I-1102 in pGEM-4Z using the BamHI and HindIII sites introduced in the primers (Meulenberg et al. 1995). The resulting plasmid was named pABV209. Two oligonucleotides located at a similar position with respect to the initiation codon (PRRSV4) and the termination codon (PRRSV5) of ORF4 of VR2332 were used to amplify ORF4 of VR2332 by means of RT-PCR as described in previous studies. The PCR fragment was digested with BamHI and partially with HindIII since ORF4 of VR2332 contains an internal HindIII site, and cloned in pGEM-4Z resulting in plasmid pABV270. Recombinant DNA techniques were performed essentially as described by Sambrook et al. (Molecular Cloning, A laboratory manual, Cold Spring Harbor Lab, Cold Spring Harbor, NY, 1989). The nucleotide sequence of VR2332 ORF4 in pABV270, determined on an automated DNA sequencer (Applied Biosystems), was identical to the published sequence (Murtaugh et al., Arch. Virol. 140; 1451-1460, 1995).

Subsequently, ORF4 of I-1102 and VR2332 were transferred to Semliki Forest virus expression vector pSFV1. pABV209 and pABV270 were digested with BamHI and HindIII (partially for pABV270), the ORF4 fragments were treated with Klenow  
5 polymerase (Pharmacia) to create blunt ends and these were ligated in the SmaI site of pSFV1, dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia). Plasmids containing ORF4 of I-1102 (pABV265) and VR2332 (pABV271) in the correct orientation were further tested for expression of  
10 the GP<sub>4</sub> protein. In addition, four different chimeric ORF4 genes of I-1102 and VR2332 were made. The nucleotide sequence of ORF4 encoding amino acids 1 through 39 of the GP<sub>4</sub> protein of VR2332 was amplified from plasmid pABV270 with  
oligonucleotides PRRSV4 and PRRSV6. The obtained fragment was  
15 digested with BamHI and SacII. This fragment was ligated in pABV209 digested with BamHI and SacII to create an in frame fusion between amino acids 1 through 39 of the GP<sub>4</sub> protein of VR2332 and 40 through 183 of the GP<sub>4</sub> protein of I-1102 in  
pABV306. The nucleotide sequence of ORF4 encoding amino acids  
20 1 through 75 of GP<sub>4</sub> of VR2332 was amplified with oligonucleotides PRRSV4 and PRRSV9 (see Table 2). This fragment was digested with KpnI and BamHI. The nucleotide sequence of ORF4 encoding amino acids 80 to 183 of the I-1102 GP<sub>4</sub> protein were amplified with PRRSV46 and PRRSV14 and the  
25 amplified fragment was digested with KpnI and BamHI. Both fragments were ligated together in pGEM-4Z digested with BamHI and HindIII, resulting in plasmid pABV308. In the same way a complementary construct was created in pABV314 consisting of the nucleotide sequence encoding amino acids 1  
30 through 79 of the I-1102 GP<sub>4</sub> protein amplified with PRRSV13 and PRRSV57 ligated to a fragment encoding amino acids 76 through 178 of VR2332, which was amplified with PRRSV10 and PRRSV5, in pGEM-4Z. A fourth chimeric construct consisted of a fragment encoding amino acids 40 through 79 of the PRRSV  
35 GP<sub>4</sub> protein fused to fragments encoding amino acids 1 through

39 and amino acids 76 through 178 of the VR2332 GP4 protein. This was achieved by ligating the BamHI/SacII ORF4 fragment of pABV270 and the SacII/HindIII ORF4 fragment of pABV314 in pGEM-4Z digested with BamHI and HindIII. This plasmid was  
 5 designated pABV325. Plasmids pABV306, pABV308, pABV314, and pABV325 were checked for the correct sequence by oligonucleotide sequencing. The chimeric ORF4 genes were transferred from pABV306, pABV308, pABV314, and pABV325 to PSFVI, identical as described above for the ORF4 genes of  
 10 VR2332 and PRRSV, resulting in pABV296, pABV305, pABV321, and pABV326, respectively (Fig. 3).

Two oligonucleotides located upstream (LV108; 5' GGAGTGGTTAACCTCGTCAAGTATGGCCGGTAAAAACCAGAGCC 3') and downstream (LV112; 5' CCATTCACCTGACTGTTTAATTAAGTGCACCCTGA 3')  
 15 of ORF7 were used to amplify and clone the ORF7 gene in pGEM-T, resulting in pABV431. The sequences and position of these and other oligonucleotides used to amplify fragments of ORF7 are listed in Table 1. In addition, four different chimeric constructs were made by PCR-directed mutagenesis. The  
 20 sequences coding for amino acids 25-26, 28-30 (site B; figure 3) were substituted for the corresponding sequences of the EAV N protein. This was accomplished by PCR amplification of ORF7 with LV108 and LV134 (5' TGGGGAATGGCCAGCCAGTCAATGACCTGTGCCGGATGTTTGGTGCAATGATAAAGTCC  
 25 3'). The mutated DNA fragment was introduced in pABV431 using the MscI and PacI site, which resulted in pABV455. The region of ORF7 encoding amino acids 51 to 67 was substituted for the corresponding region of LDV ORF7. pABV431 was digested with EcoNI and ClaI and ligated to a PCR fragment produced with  
 30 primers LV98 (5' CCAGCAACCTAGGGGAGGACAGGCCAAAAAGAAAAAGCAGCCGAAGCTACATTTTCCCATG GCTGGTCCATCTGAC 3') and LV99 (5' CGTCTGGATCGATTGCAAGCAGAGGGAGCGTTCAGTCTGGGTGAGGACGTGCCGGAGGTCA GATGGACCAGCC 3'), digested with the same enzymes. This  
 35 plasmid was designated pABV463. The region of ORF7 encoding amino acids 80 to 90 was substituted for the corresponding

region of the LDV ORF7 gene. The ORF7 gene of LV was mutated in a PCR with primers LV101 (5' GCTTGCAGGCGCCCGTGACGCTTTTCAATCAAGGCGGAGGACAGGCGTCGCTTTCATCCA3') and LV112. The obtained fragment was digested with *NarI* and *PacI* and ligated to pABV431 digested with *ClaI* and *PacI*. This resulted in pABV453. Finally, the region encoding the C terminal part of the N protein (amino acids 111-128) was replaced for a sequence encoding the corresponding amino acids of the N protein of LDV. The ORF7 gene was amplified with primers LV108 and LV102 (5' ATGTCCCGGGCTAAGCGGCGGAGGAATTAGCAGAAGCGTTAATCAGGCGCTGTGTAGCAGCAACCGGCAG 3') and cloned in the pGEM-T vector, which resulted in pABV456. The wild type and mutated ORF7 genes were excised from pABV431, pABV453, pABV455, and pABV463 by digestion with *PacI* (blunt ended) and *HpaI* and from pABV456 by digestion with *HpaI* and *SwaI*. These genes were subsequently inserted in the dephosphorylated *SmaI* site of Semliki forest virus expression vector pSFV1. Plasmids pABV470, pABV460, pABV462, pABV518 and pABV471 containing the respective ORF7 genes in the correct orientation were further tested for expression of the N protein. In vitro transcription and transfection of Semliki forest virus ORF7 RNA was as described above for the SFV-ORF4 constructs.

To clone the ORF4 genes of seven different European isolates macrophages were infected with NL1, NY2, DEN, FRA, SPA1, SPA2, and LUX, and RNA was isolated as described by Meulenberg et al. (1993). The ORF4 genes were amplified by means of RT-PCR with oligonucleotides PRRSV13 and PRRSV14, and cloned with *BamHI* and *HindIII* in pGEM-4Z. For each strain, the nucleotide sequence of ORF4 of two clones derived from two independent PCRs was determined. The protein sequences derived from the nucleotide sequence were aligned using the multiple sequence alignment program CLUSTAL of PCGene (Intelligenetics Tm).



In vitro transcription and transfection of SFV-ORF4 RNA.

pSFV1 plasmids containing different ORF4 constructs were linearized by digestion with SpeI and transcribed in vitro. The synthesized RNA was transfected to BHK-21 cells in 15 mm wells of twentyfour-well plates using lipofectin. Cells were fixed with ice-cold 50% (v/v) methanol/acetone and the GP4 protein expressed by the different ORF4 constructs was stained with MAbs in the immunoperoxidase monolayer assay (IPMA). To analyze the ORF4 expression products by immunoprecipitation, 10<sup>7</sup> BHK-21 cells were transfected with 10 µg in vitro transcribed SFV-ORF4 RNA by electroporation. The electroporated cells were plated in three 35 mm wells of six-well plates and 18 h after transfection cells were labeled.

15

#### Pepscan method

A complete set of overlapping nonapeptides or dodecapeptides was synthesized from amino acids derived of the ORF4 or ORF7 sequence of PRRSV, as was determined previously (Meulenberg et al., 1993). The synthesis of solid-phase peptides on polyethylene rods and immunoscreening with an enzyme-linked immunosorbent assay (ELISA) type of analysis were carried out according to established PEPSCAN procedures (Geysen et al., PNAS, 81, 3998-4002, 1984).

25

#### RESULTS

We have previously described a panel of neutralizing MAbs that reacted with a 31 to 35 kDa protein of PRRSV, designated GP4, and a panel of MAbs reactive with the N protein, by Western immunoblot analysis. GP4 was shown to be a structural glycoprotein encoded by ORF4, N was shown to be the nucleocapsid protein encoded by ORF7. In immunoprecipitation experiments with GP4 specific MAbs, the GP4 protein derived from lysates of cells infected with PRRSV, migrated as a discrete band of 28 kDa together with a light smear of somewhat higher apparent molecular weight. The

35

MAbs immunoprecipitated a diffuse (glycosylated) GP4 protein of about 31 kDa from the extracellular medium of PRRSV-infected but not from the extracellular medium of mock-infected cells.

5

Identification of the neutralizing domain in GP4.

We have demonstrated earlier that MAbs specific for the GP4 protein recognized I-1102 but not the US isolate VR2332 (van Nieuwstadt et al., 1996). In order to identify the  
10 binding domain of the neutralizing MAbs in the GP4 protein, we made fusion proteins of the GP4 protein of I-1102 and VR2332. These proteins were expressed in the Semliki Forest virus expression system, developed by Liljeström et al. (Biotechnol, 9, 1356-1362, 1991). First, ORF4 of I-1102 was  
15 cloned in pSFV1 resulting in plasmid pABV265 (Fig. 1). RNA transcribed from pABV265 was transfected to BHK-21 cells and 24 h after transfection cells were positively stained with the panel of fifteen neutralizing MAbs. The MAbs did not react with BHK-21 cells transfected with pSFV1 -RNA. The  
20 recombinant GP4 protein was immunoprecipitated with MAb 126.1 from L-[<sup>35</sup>S]-methionine labeled BHK-21 cells transfected with pABV265 RNA. It had a similar size as the authentic GP, protein synthesized in CL2621 cells infected with I-1102 and also contained PNGaseF and EndoH sensitive N-glycans. The GP4  
25 protein of VR2332 was also cloned in pSFV1, but this protein was not recognized by the MAbs upon expression in BHK-21 cells (Fig. 1). To further localize the region in the GP4 protein recognized by the MAbs, four chimeric genes of ORF4 of I-11022 and VR2332 were constructed in pSFV1 (Fig. 1). RNA  
30 transcribed from plasmids pABV296, pABV305, pABV321, and pABV326 was transfected to BHK-21 cells and the reactivity of the expressed proteins with the GP4-specific MAbs was tested in IPMA. The reaction pattern of these fifteen MAbs was identical, and indicated that these MAbs were directed to a  
35 region of 40 amino acids in the GP4 protein; The expression

product of pABV326, consisting of amino acids 40 through 79 derived from the GP<sub>4</sub> protein of isolate CNCM I-1102 and surrounded by sequences derived from the VR2332 GP<sub>4</sub> protein was still recognized by the panel of MABs. To ensure that the different GP<sub>4</sub> proteins, especially those which were not recognized by the MABs, were properly expressed in BHK21 cells, they were immunoprecipitated from lysates of BHK-21 cells that were transfected with RNA transcribed in vitro from plasmids pABV265, pABV271, pABV296, pABV305, pABV321, and pABV326. Immunoprecipitation was carried out with porcine anti-PRRSV serum 21, MAb 126.1, and anti-peptide sera 698 and 700. Serum 700 is directed against amino acids 106-122 of the PRRSV GP<sub>4</sub> protein of isolate CNCN I-1102, a sequence which is identical in the GP<sub>4</sub> protein of isolate ATCC-VR2332, apart from amino acid 121. Therefore all GP<sub>4</sub> proteins were immunoprecipitated with serum 700. They were indistinguishable in size, when analyzed by SDS-PAGE, except for the GP<sub>4</sub> proteins expressed by pABV305 and pABV271, which migrated slightly faster. This is most likely due to the deletion of 4 amino acids in the VR2332 sequence relative to the I-1102 sequence, between amino acids 62-64 (Fig. 3). The complete set of GP<sub>4</sub>-specific MABs recognized the GP<sub>4</sub> proteins expressed from pABV265, pABV296, pABV321, pABV326, but not those expressed from pABV305 and pABV271, which confirmed the results obtained by IPMA (Fig. 3). Serum 698 had the same reaction profile as the MABs. Serum 698 is directed against amino acids 62 to 77 of GP<sub>4</sub> of PRRSV, which are located within the now identified neutralization domain of the GP<sub>4</sub> protein. This region is highly heterogeneous in VR2332 ORF<sub>4</sub>, and therefore the expression products containing the VR2332 sequence in this region were not recognized by this serum. However, neutralizing polyclonal pig sera recognize the I-1102 GP<sub>4</sub> protein and the chimeric GP<sub>4</sub> proteins and the VR2332 GP<sub>4</sub> protein, indicating that in porcine anti-PRRSV sera a variety of neutralizing antibodies that are directed against

the neutralizing site formed by amino acids 40 to 79 of the GP<sub>4</sub> protein are present.

Peptscan of the ORF4 and ORF7 protein

5        Since the fifteen MAbS reactive with the ORF4 protein all reacted with the GP<sub>4</sub> protein in western blot analysis, they were expected to recognize a linear epitope in a region spanning amino acids 40 through 79 of GP<sub>4</sub> of isolate I-1102. To further map the binding region of the MAbS, a PEPSCAN  
10 analysis was performed using overlapping nonapeptides or dodecapeptides in this region. Peptides were considered to represent antigenic sites if peaks in such a set reproducibly amounted to more than twice the background. MAbS 122-29, 122-30, 122-66, 122-71, 130-7, 138-28 reacted positively with one  
15 specific antigenic site consisting of amino acids 59 through 67 (SAAQEKISF) (Fig. 2). MAb 122-12 reacted only weakly to this antigenic site, whereas the remaining 7 MAbS were negative in the PEPSCAN analysis. Polyclonal pig sera also identified this site in PEPSCAN. Neutralizing serum 21, taken  
20 at week 6 after infection of pig 21 with PRRSV reacted strongly and broadly with the site and its flanking regions. In addition, neutralizing polyclonal pig sera (val2 and val4), taken at 54 days after vaccination with PRV-ORF4 vector virus and at slaughter at 30 days after challenge at  
25 day 54 with PRRSV, reacted strongly and more broadly with the neutralization site identified in PEPSCAN. In isolate I-1102, the core sequence of the neutralization site comprises the aa sequence SAAQEKISF located from aa position 59-67. In other isolates the core sequence can be  
30 found at or around the corresponding aa position, which is an amino acid sequence corresponding to a neutralization site of protein GP<sub>4</sub>, comprising for example sequences such as SAAQEEISF, or STAQENISF or STAQENIPF or SEESQSVT or SASEAIR or SASEAFR or PAPEAFR or PAPEAIR or SAFETFR or STSEAFR, but  
35 it is to be expected that other isolates of PRRSV have corresponding but slightly differing core sequences of the

neutralization site located at or around the aa position corresponding to aa 59-67 of the ORF 4 amino acid sequence of the I-1102 isolate of PRRSV. Also, artificial changes that maintain the antigenicity and thus functionality of the above  
5 core sequences can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Also, as is clearly demonstrated by the much broader reactivity in PEPSCAN of the neutralizing polyclonal sera, aa sequences comprising aa core sequences and aa sequences flanking the  
10 core sequences of the various PRRSV isolates in addition constitute the neutralization site on the ORF4 protein of PRRSV. Especially sequences located at positions corresponding to about aa 40 to 79 constitute the neutralization site (Fig 1). Again, artificial changes that  
15 maintain the antigenicity and thus functionality of the above antigenic sites can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Also, considering the broad reactivity of the polyclonal neutralizing sera val2 and val4 (Fig 2), aa sequences located  
20 at positions corresponding from about aa 52 to 75 more specifically constitute a broadly reactive neutralization site.

The Mabs directed against the ORF7 protein reacted in four different groups in PEPSCAN, group A(4), B(2), C(3) and D(1).  
25 Group 1(D) (in which among others Mabs 122.17, 130.3, 130.4, 131.7, WBE1, WBE4, WBE6, SDOW17 and comprising conserved and non-conserved reactive sites) reacted with a conformational epitope not detectable in PEPSCAN. Group 2(B) (in which among others 125.1, 126.9, NS95 and NS99 and reactive with all  
30 isolates of PRRSV tested, thus identifying a conserved antigenic site) identifies a core sequence VNQLCQLLGA (found in isolate I-1102 from aa position 22 to about 32) or VNQLCQMLGK. Group 3(C) (in which among others Mab 126.15 and mainly reactive with strains of PRRSV isolated in Europe,  
35 thus identifying a differentiating antigenic site) identifies a core sequence PRGGQAKKKK (found in isolate I-1102 from aa position 41 to about 50) or PRGGQAKRKK or PRGGQAKKRK or

GPGKKNNKKKN or GPGKKNNKKKT or GPGKKNNRKKN or GPGKKFKKKKN or GPGKKIKKKKN or GPGQINKKIN. Group 4 (A) (in which among others Mab 138.22 and mainly reactive with strains of PRRSV isolated in Europe, thus identifying a differentiating antigenic site) identifies a core sequence MAGKNQSQKK (found in isolate I-1102 from aa position 1 to about 10) or MPNNNGKQTE or MPNNNGKQPK or MPNNNGKQOK or MPNNNGKQON or MPNNNGKQOK or MPNNNGRQOK. Also, artificial changes that maintain the antigenicity and thus functionality of the above antigenic sites in the N protein can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Although group 1 does not constitute linear epitopes, comparison of PRRSV aa sequences with LDV sequences shows that conformational epitopes (which vary greatly among the various isolates) can be found at positions corresponding to those found in isolate I-1102 from aa position 51 to about 68 (in isolate I-1102 aa sequence PKPHFPLAEDDIRHHL) or from 79 to about 90 (in isolate I-1102 aa sequence SIQTAFNQGAGT) or from 111 to 124 (in isolate I-1102 aa sequence HTVRLIRVTSTSAS). Also, artificial changes that maintain the antigenicity and thus functionality of the above conformational epitope sites in the N protein can easily be introduced by the average expert skilled in the art of peptide design and synthesis, especially with information gathered by sequence comparison of PRRSV isolates, and by comparison with N protein sequences of other Arteriviridae. This was determined in expressing chimeric LDV/PRRSV ORF7 proteins in the SFV expression system (done as above for ORF4) and determining their reactivity with Mabs from group 1.

30

#### Chimeric N proteins

Domain D was further mapped with constructs of ORF7 expressing chimeric N proteins. Since 6 out of 10 Mabs directed against domain D recognized both European and North American isolates of PRRSV, the regions which were most conserved between the N protein of LV and the North American

35

prototype VR2332 (Fig. 4) were mutated. The nucleotide sequence coding for amino acids 51 to 67, 80 to 90, and 111 to 128 was substituted for a sequence that codes for the corresponding amino acids of LDV (Fig. 4). For completion, site B (amino acids 25-30) that is also conserved in European and North American isolates, was mutated. Since the amino acid sequence of the LV N protein was very similar to that of the LDV N protein in site B, this region of the LV N protein was substituted for a region encoding the corresponding amino acids of the EAV N protein (Fig. 4). When the mutated and wild type N proteins were expressed in BHK-21 cells using the Semliki forest virus expression system, and they were tested with the N-specific MABs in IPMA, the D-specific MABs reacted identically (Table 1). Their binding was disrupted by mutations between amino acids 51-67 and 80-90, but not by mutations between amino acids 111-128 or amino acids 25-30 (site B). As was expected, the N proteins with LDV sequences between amino acids 51-67 and 80-90 were still stained by MABs directed against sites A, B, and C. However, the number of cells that were stained and the brightness of this staining was less than that observed for the wildtype N protein and the N proteins mutated in amino acids 25-30 (site B) or in amino acids 111-128 (Table 1). This was most likely due to a lower expression of the N proteins containing mutations between amino acids 51-67 or 80-90, since a lower yield of these mutant N proteins compared to the other N proteins was also obtained when equal amounts of transcripts were translated in vitro (data not shown). As was expected, the N protein that contained EAV sequences in site B was not recognized by MABs mapped to site B (by pepscan analysis), but was still recognized by MABs that mapped to sites A, C, or domain D. These data indicate that the epitopes mapped to domain D are conformation-dependent and consist (partially) of amino acids 51-67 and 80-90.

Sequence analysis of the GP<sub>4</sub> protein of different PRRSV strains.

To analyze whether the major antigenic neutralization site, recognized by the GP<sub>4</sub>-specific antibodies, was conserved among different PRRSV isolates, the reactivity and neutralizing activity of the MAb was further tested on seven different European strains. The results indicated that these MAb recognized and neutralized another Dutch strain NL1 and an English strain NY, but not Danish isolate DEN, two Spanish strains SPA1 and SPA2, a French isolate FRA, and LUX isolated in Luxembourg. Therefore we were interested in the amino acid sequence, in the region of the neutralization site of the GP<sub>4</sub> protein of these isolates. The ORF4 genes were cloned by means of RT-PCR using primers derived from the PRRSV sequence and the nucleotide sequence was determined. The amino acid sequence of the GP<sub>4</sub> protein of the different isolates derived from this nucleotide sequence were 86 to 97% identical with that of I-1102. The alignment of these amino acid sequences showed that the neutralization site (amino acids 40 through 79) is much more divergent than the remaining part of the protein. In this region, especially the amino acid sequences of strains DAN, SPA1, SPA2 and FRA are different. This is in line with the finding that these strains are not neutralized by the I-1102 specific MAb and further confirms that this site is not highly conserved among European isolates. Another region of higher heterogeneity was observed in the N-terminal part of the GP<sub>4</sub> protein. Comparison of the amino acid sequence of the PRRSV GP<sub>4</sub> protein and that of VR2332 and other North American strains shows that the latter are also heterogeneous in the neutralization site of the protein. Alignment of the amino acid sequences results in the introduction of a gap in the neutralization site of the North American isolates (Fig. 3), which is in agreement with the observation that none of these isolates are recognized by the MAb. Overall, a higher diversity was observed among the



sequences of the American isolates than among the sequences of the European isolates.

This is in line with the features characteristic for typical viral envelope, identified e.g. in the amino acid sequence of GP4.

The potential of the neutralization site for vaccine development is of great importance in view of the heterogeneity of the neutralization site. Comparison of the amino acid sequence of the GP4 proteins of different European strains indicated that the neutralization site was much more variable than other parts of the protein, suggesting that this site is susceptible to immunoselection. Comparison of the neutralization site sequences of European and North American strains displayed a gap of 4 amino acids in the North American sequences with respect to the European, further illustrating the large amino acid variability of the now identified neutralization site of PRRSV.

The neutralization site in the GP4 protein described here, is the first site identified for Lelystad virus. For two other arteriviruses, EAV and LDV, the neutralizing MAbs that were isolated, were all directed against the G<sub>1</sub>/VP3 protein encoded by ORF5 (Deregt et al, 1994; Glaser et al, 1995; Balasuriya et al, 1995; Harty and Plagemann, 1988).

Using neutralization-escape mutants, the neutralization site of EAV was mapped to specific amino acids in the ectodomain of G<sub>1</sub>.

Similar sequence comparisons were done for the ORF7 protein of PRRSV (fig. 4) further illustrating the large amino acid variability of the now identified antigenically conserved site and non-conserved sites of PRRSV. In this work we have identified four distinct antigenic sites in the N protein of PRRSV. Three sites, designated A, B, and C contain linear epitopes and these were mapped between amino acids 2-12, 25-30, and 40-46, respectively. In contrast, the fourth site, designated domain D, contains conformation-dependent epitopes that are (partially) composed of amino acids 51-67

and 80-90. Sites A and C contain epitopes that are conserved in European but not in North American isolates of PRRSV, site B contains epitopes that are conserved in European and North American isolates of PRRSV, whereas site D contains both  
5 epitopes that are conserved and not conserved in European and North American isolates of PRRSV. The conserved sites in the N protein described here, are of great importance in the development of diagnostic tests aimed at unequivocal diagnosis of PRRSV infections, these tests should avoid  
10 employing non-conserved sites thereby avoiding false-negative results. In addition, knowledge about the various non-conserved sites is highly valuable in the development of differentiating tests that can e.g. discriminate vaccinated pigs from pigs infected with wild type isolates of PRRSV.

## Legends.

Fig. 1. Schematic diagram of G proteins expressed in pSFV1 and their reactivity with GP<sub>4</sub>-specific MAbs. The names of the plasmids containing the different ORF4 genes are indicated. Open bars represent the amino acid sequences derived from the G protein encoded by ORF4 of PRRSV, black bars represent amino acid sequences derived from the G protein encoded by ORF4 of VR2332. The numbers of the amino acids are indicated above the bars. The genes were first inserted in PGEM-4Z and then transferred to pSFV1, as described in detail in the Materials and methods section. The complete set of 14 GP<sub>4</sub>-specific MAbs reacted identically with the different constructs in IPMA and the reactivity is indicated as positive (+) and negative (-).

Fig. 2. Pepscan analysis of GP<sub>4</sub>-specific MAbs and polyclonal sera with overlapping 12-mer peptides covering residues 25 to 94 of GP<sub>4</sub>. The scan of MAbs 130.7 and MAb 138.28 which recognize four consecutive peptides is shown (2A). Five other MAbs (122.29, 122.30, 122.66, 122.71 and 138.28) exhibited similar specificity. The scans of polyclonal sera from two pigs before immunization (va12-0 and va14-0), after immunization with a pseudorabies virus vector expressing ORF4 (va12-54 and va14-54) and after subsequent challenge with PRRSV (va12-s1 and va14-s1) are shown (2A and 2B), and the scan of polyvalent porcine anti-LV serum 21 (va21) is shown (2D). The amino acid sequence of the reactive peptides is shown with the core of common residues boxed.

Fig. 3. Alignment of amino acid sequences of GP<sub>4</sub> (A) and N (B) proteins of various PRRSV strains. Only the amino acids which differ from the I-1102 sequence are shown. The core peptide sequences recognized by MAbs and/or polyclonal sera in pepscan analysis are underlined.

Fig. 4. Location of antigenic binding sites in the N protein sequence and comparison of the N protein sequence with those of North American strain VR2332 and LDV. Antigenic sites A, B, C, and domain D are shown in shadow. Sites A, B, and C were identified in pepscan analysis, site D was identified by construction of chimeric N proteins. The amino acid sequences of LV that were substituted for the corresponding amino acid sequences of LDV in order to map domain D are underlined. The amino acids of the N protein of EAV that were inserted between amino acids 25-30 to mutate site B are shown below the LDV sequence. Identical amino acids are connected with vertical bars.

Table 1. Staining of chimeric N proteins expressed by Semliki forest virus in BHK-21 cells in IPMA

Plasmid	Mutation	Staining with MAbs in IPMA			
		138.22	125.1/126.9/NS95/NS99	126.15	122.15/130.2/130.4/131.7/131.9/WBE1/WBE4/WBE5/WBE6/SDOW17
		A	B	C	D
pABV470	-	+++	+++	++	+++
pABV462	25-30	+++	-	++	+++
pABV518	51-67	++	++	+	-
pABV460	80-90	++	++	+	-
pABV471	111-124	+++	+++	++	+++

Table 2. Sequence of primers used in PCR to clone the ORF4 genes of LV and VR2332 and chimeric ORF-4 genes in plasmid vectors pGEM-4Z and pSFV1

	Name	Sequence <sup>a</sup>	Incorporated restriction site
5			
	LV13	5' GGCAATTGGATCCATTGGA 3'	<i>Bam</i> HI
	LV14	5' AGAAGCAAGCTTGC GGAGTC 3'	<i>Hind</i> III
10	LV46	5'GCCGTCCGGTACCCCTCAGTACAT 3'	<i>Kpn</i> I
	LV57	5'ATGTACTGAGGGGTACCGACGGC 3'	<i>Kpn</i> I
	PRRSV4	5' GGCAATTGGATCCACCTAGAATGGC 3'	<i>Bam</i> HI
	PRRSV5	5' GCGAGCAAGCTTCCGCGGTCAAGCATTCT 3'	<i>Hind</i> III
	PRRSV6	5'CTTGCCGCCCGCGGTGGTGTG 3'	<i>Sac</i> II
15	PRRSV9	5' ACAGCTGGTACCTATCGCCGTACGGCACTGA 3'	<i>Kpn</i> I
	PRRSV10	5' GCGATAGGTACCCCTGTGTATGTTACCAT 3'	<i>Kpn</i> I

20 <sup>a</sup> The underlined nucleotides in these primers are mutated with respect to the original sequence to create restriction sites or overhanging sequences or to avoid long stretches of one particular nucleotide. The restriction sites in the primers are shown in italics.

CLAIMS

1. A peptide eliciting neutralizing antibodies comprising an amino acid sequence of at least 7 to 40 amino acid residues derived from an amino acid sequence corresponding to a neutralization site of protein GP4 of PRRSV.
- 5 2. A peptide eliciting neutralizing antibodies according to claim 1 comprising an amino acid sequence wherein amino acid residues have been replaced conventionally or under guidance of replacement mapping.
3. A peptide according to claim 1 or 2 wherein said
- 10 neutralization site comprises the amino acid residues located at about amino acid position 40 to 79 of protein GP4 of PRRSV isolate I-1102.
4. A peptide according to any of claims 1 to 3 wherein said neutralization site comprises the amino acid residues located
- 15 at about amino acid position 52 to 75 of protein GP4 of PRRSV isolate I-1102.
5. A peptide according to any of claims 1 to 4 wherein said neutralization site comprises the amino acid residues located at about amino acid position 59 to 67 of protein GP4 of PRRSV
- 20 isolate I-1102.
6. A peptide according to any of claims 1 to 5 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of SAAQEKISF, SAAQEEISF, STAQENISF, STAQENIPF, SEESQSVT, SASEAIR, SASEAFR, PAPEAFR, PAPEAIR,
- 25 SAFETFR and STSEAFR.
7. A peptide eliciting antibodies which react with at least two different PRRSV isolates comprising an amino acid sequence of at least about 5 to about 15 amino acid residues derived from an amino acid sequence corresponding to a
- 30 conserved site of protein N of PRRSV.
8. A peptide eliciting antibodies which react with at least two different PRRSV isolates according to claim 7 comprising an amino acid sequence wherein amino acid residues have been

replaced conventionally or under guidance of replacement mapping.

9. A peptide according to claim 7 or 8 wherein said conserved site comprises the amino acid residues located at about amino acid position 22 to 32 of protein N of PRRSV isolate I-1102.

10. A peptide according to any of claims 7 to 9 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of VNQLCQLLGA and VNQLCQMLGK.

11. A peptide eliciting antibodies which are capable of distinguishing between at least two different PRRSV isolates comprising an amino acid sequence of at least 5 to 15 amino acid residues derived from an amino acid sequence corresponding to a non-conserved and differentiating site of protein N of PRRSV.

12. A peptide eliciting antibodies which are capable of distinguishing between at least two different PRRSV isolates according to claim 11 comprising an amino acid sequence wherein amino acid residues have been replaced conventionally or under guidance of replacement mapping.


13. A peptide according to claim 11 or 12 wherein said non-conserved site comprises the amino acid residues located at about amino acid position 41 to 50 of protein N of PRRSV isolate I-1102.

14. A peptide according to any of claims 11-13 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of PRGGQAKKKK, PRGGQAKRKK, PRGGQAKKRK, GPGKKNKKKN, GPGKKNKKKT, GPGKKNRKKN, GPGKKFKKKN, GPGKKIKKKN and GPGQINKKIN.

15. A peptide according to claim 11 wherein said non-conserved site comprises the amino acid residues located at about amino acid position 1 to 10 of protein N of PRRSV isolate I-1102.

16. A peptide according to claim 11, 12 or 15 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of MAGKNQSQKK, MPNNNGKQTE, MPNNNGKQPK, MPNNNGKQOK, MPNNNGKQON, MPNNNGKQOK and MPNNNGRQOK.



17. A peptide eliciting antibodies directed against a conformational epitope site or protein N of PRRSV, said peptide having a length of from 5 to 15 amino acid residues, said amino acid residues corresponding to a conformational stretch located from about amino acid position 68 to 79 or from 79 to 90 or from 111 to 124 of protein N of Lelystad virus isolate I-1102.
18. A peptide according to claim 17 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of PKPHFPLAAEDDIRHHL, SIQTAFNQGAGT and HTVRLIRVTSTSAS. 
19. An immunogenic composition for inducing neutralizing antibodies corresponding to a neutralization site of protein GP4 of Lelystad virus comprising at least one peptide according to any of claims 1 to 6.
20. A vaccine for the prophylaxis of PRRS infections comprising a peptide according to any of claims 1 to 6 or an immunogenic composition of claim 19, together with a suitable adjuvant or carrier for administration to an animal.
21. A synthetic antibody reactive with a peptide according to any of claims 1-18.
22. A diagnostic test kit for the detection or identification of antibodies directed against a PRRSV isolate comprising at least one peptide of any of claim 1 to 18 together with suitable means for detection.
23. A diagnostic test kit for the detection or identification of antibodies directed against or antigen derived from a PRRSV isolate comprising an antibody according to claim 21.
24. Use of a vaccine according to claim 20 or a diagnostic test or kit according to claim 22 or 23 to reduce the occurrence of PRRS in a pig herd.
25. Use of a diagnostic test kit according to claim 22 or 23 to test for the occurrence of PRRS in a pig or pig herd.
26. Use of a vaccine according to claim 20 or a diagnostic test or kit according to claim 22 or 23 in eradication programs to reduce or terminate the occurrence of PRRS in a pig herd.

27. A method for testing for the occurrence of PRRS in a pig or a pig herd comprising the use of a diagnostic test kit according to claim 22 or 24.

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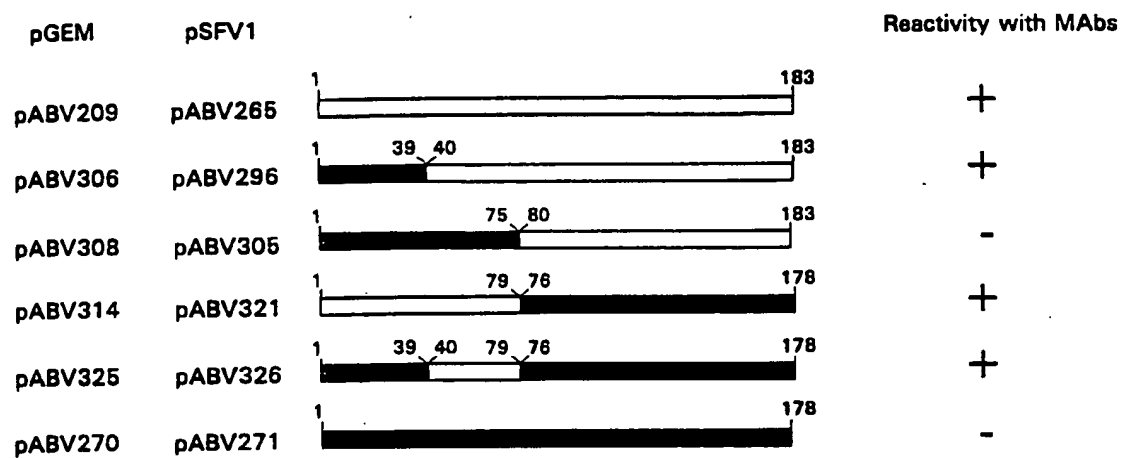


Fig. 1

Fig.2A

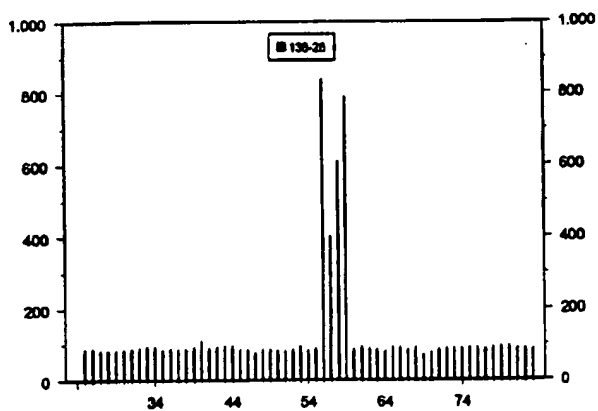
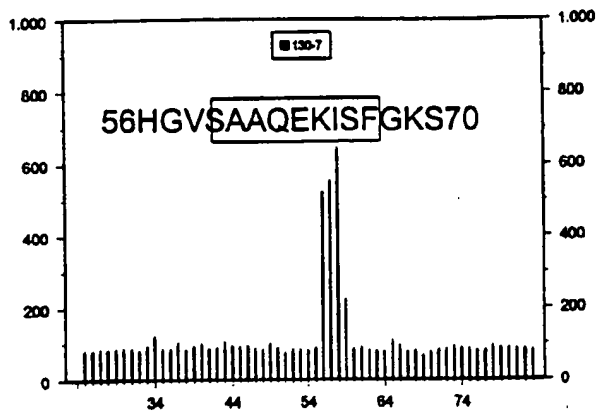


Fig.2B

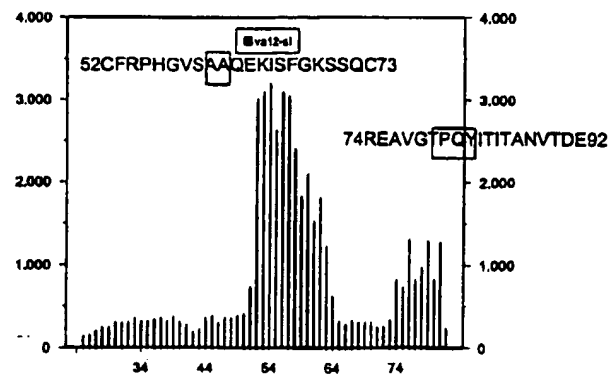
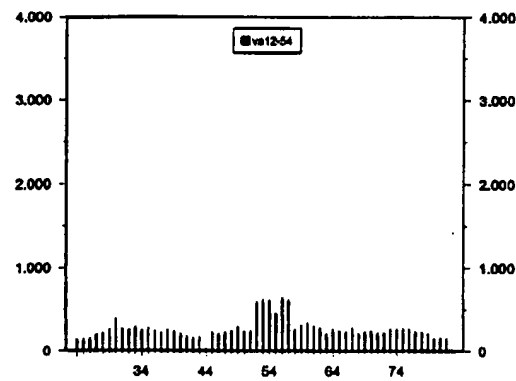
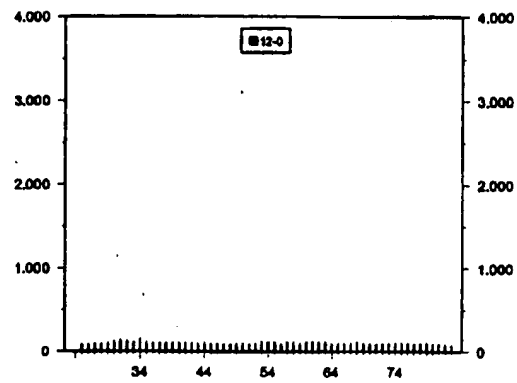


Fig.2C

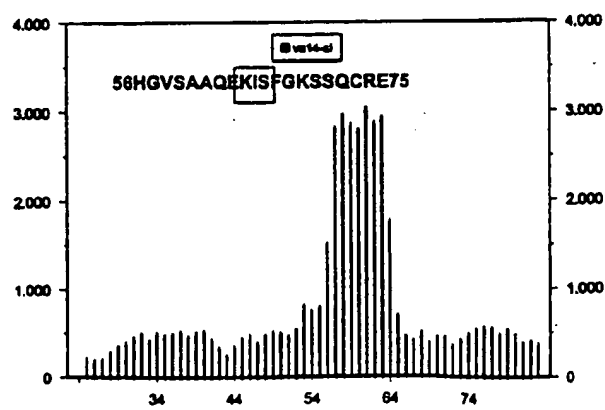
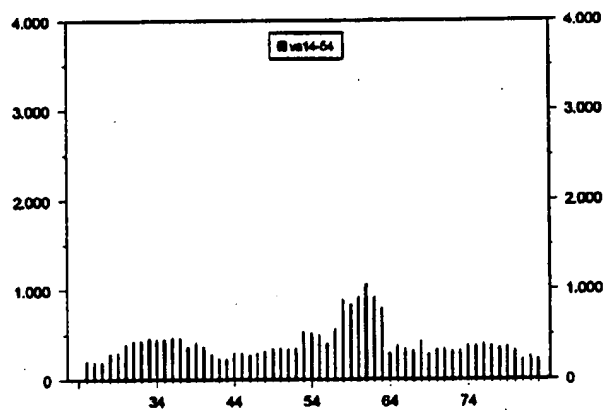
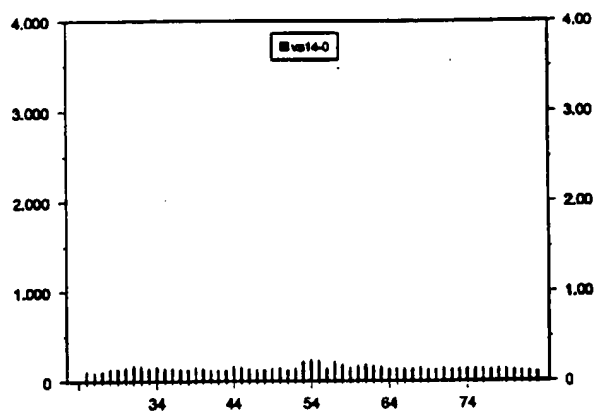


Fig.2D

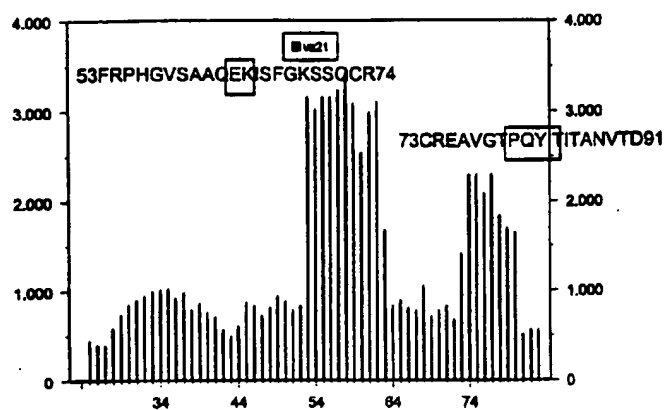


Fig. 3A Alignment of amino acids of GP<sub>1</sub> of PRRSV isolates

I-1102-4	MAAATLFFLAGAQHIMVSEAFACKPCFSTHLSDIETNTTAAAGFMVLQDI	50
NL1-4		50
NY2-4	L	50
PRRSV10-4	L	50
LUX-4	L	50
SPA1-4	I S F	50
FRA-4	I L F	50
DEN-4	I L L	50
VR2332-4	SSL LVV FKCLL Q SS A K S A	50
IA1-4	SL L V FKCLL Q SS A K S A E	50
KS1-4	SL LMV FKCLL Q SS A K SSV	50
MN1-4	SL LMV FKCLL Q SS A K S A	50
IA6-4	SL L V FKCLL Q SS A K S A	50
IL1-4	SL L V FKCFV Q SS A K S A	50
NE1-4	SSL LMV FKCLL Q SS A K N A E	50
SG1-4	SL L V FKCLL Q NQ SS E K G A E	50
VR2385-4	SL L V FKCLL Q SS K G A	50
KY1-4	SL LMV FKCLL Q SS A K S A	50
MO1-4	SL LMV FERLL Q SS A K G A E	50

\*\*\* \*\*

I-1102-4	NCFRPHGVSAAQEKISFGKSSQCREAVGTPQYITITANVTDESILYNADL	100
NL1-4		100
NY2-4		100
PRRSV10-4		100
LUX-4	L R E	100
SPA1-4	L T N P P I	100
FRA-4	L T N P P I	100
DEN-4	T A A EESQSVT N P I	100
VR2332-4	S L HRDSASE---AIR IP T I V V N HSS	96
IA-4	S L HRNSASE---AIR VP T I V N HSS	96
KS1-4	S L HRNSASE---AIR VP Y T I V N HSS	96
MN1-4	S L HRNSASE---AIR IP A I V N HSS	96
IA6-4	S L HRDSASE---AIR P T I V N HSS	96
IL1-4	S L HRDSASE---AIR IP T I V N HSS	96
NE1-4	S L HRNPAPPE---A R IP T I V N HSS	96
SG1-4	S L HRNPAPPE---AIR VP T I V SV N HSS	96
VR2385-4	S L HRNSASE---AIR VP T I V V N HSS	96
KY1-4	S L HRDSASE---T R VP T I V V N HSS	96
MO1-4	S L HRDSTSE---AFR VP T I V V N HSS	96

\* \* \* \* \*



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★ ★★ ★★ ★★ ★★ ★★ ★★ ★★ ★★

Fig. 3B Alignment of amino acid sequences of N of PRRSV isolates

I-1102-7	<u>MAGKNOSOKKKKSTAPMGNGQPVNOLCOLLGAMIKSQ---</u>	<u>ROQPRGGQAK</u>	47
PRRSV10-7			47
VR2332-7	PNN GK TEE ----K D	M KI AQ NQS GKGP KKN	46
NE1-7	PNN GK TEE ----R D	M KI AQ NQS GKGP KKN	46
IL1-7	PNN GK P E ----K D	M KI AQ NQS GKGP KKN	46
IA1-7	PNN GK Q ----K D	M KI AQ NQS GKGP KKN	46
IA6-7	PNN GK QN ----K D	M KI AQ NQS GKGP KKN	46
KS1-7	PHN GK Q R ----K D	M KI AQ NQS GKGP KKF	46
MN1-7	PNN GK Q R ----K D	M KI AQ NQS GKGP KKN	46
SG1-7	PNN GR Q ----K DC	M KI AQ NQS GKGP KKN	46
MO1-7	PNN GR Q ----K D	M KI AQ NQS GKGP KKI	46
KY1-7	PHN GR Q ----K D	M KI AQ NQS GKGP QIN	46
ISU3927-7	PNN GK Q ----K D	M KI AQ NQS GKGP KKN	46
ISU55-7	PNN GK Q ----K D	M KI AQ NQS GKGP KKN	46
ISU1894-7	PNN GK Q R ----K D	M KI AQ NQS GKGP KKN	46
VR2385-7	PNN TGK Q R ----K D	M KI AH NQS GKGP KKN	46
IAF-7	PNN GR Q ----K D	M KI AQ NQS GKGP KKN	46
	* . . . *	* . . . . . *	

I-1102-7	<u>KKKPEKPHFPLAAEDDIRHHLTQTERS</u>	<u>SLCQSIQTAFNOGAGTASLSSSG</u>	97
PRRSV10-7		P	97
VR2332-7	N	T V F PS Q S	CT D
NE1-7	T	T V F PS Q S	CT D
IL1-7	N	T V F PS Q S	CT D
IA1-7	N	T V F PS Q S	CT D
IA6-7	N	T V F PS Q S	CT D
KS1-7	N	T V F PS Q S	CT D
MN1-7	N	T V F PS Q S	ICI D
SG1-7	N	T V V F PS Q S	CT D
MO1-7	N	SI FV F PS Q S	CT D
KY1-7	IN V YS VT	V F PS Q S	CT D
ISU3927-7	N	T V F PS Q S	CT D
ISU55-7	N	T V F SG Q S	CT D
ISU1894-7	N	T V F PS Q S	CT D
VR2385-7	N	T V F PS Q S	CT D
IAF-7	N	T V F PS Q S	CT D
	* . * . . *	* . . . . *	* . . . . *

I-1102-7	<u>KVSFQVEFMLPVAHTVRLIRVTST</u>	<u>SASQGAS</u>	128
PRRSV10-7			128
VR2332-7	RI YT S TH	ASPSA----	123
NE1-7	RI YT S TH	ASPSA----	123
IL1-7	RI YT S TH	APSSA----	123
IA1-7	RI YT S TH	ASPSA----	123
IA6-7	RI YT S TH	APPSA----	123
KS1-7	RI YT S TH	ASPSA----	123
MN1-7	RI YT S TH	VSPSA----	123
SG1-7	RI YT S TH	ASPSA----	123
MO1-7	RI YT S TH	ASPSA----	123
KY1-7	RI YT S TH	ASPSA----	123
ISU3927-7	RI YT S TH	APPSA----	123
ISU55-7	RI YT S TH	APPSA----	123
ISU1894-7	RI YT S TH	ASPSA----	123
VR2385-7	RI YT S TH	ASPSA----	123
IAF-7	RI YA S TH	ASPSA----	123
	... * . . *	* . . . . *	....

Fig. 4

	A	B	C	
VR2332	MPNNNGKQTEEK----	KGDGQPVNQLCOMLGKIIAQQNQSRGKGP	GKKN	45
LV	MAGKNQSQKKKKSTAPMGNGQPVNQLCOLL	GAMIKSQ---	RODPREGGQA	46
LDV	M-----SQNKKKSGQNKGANQ---	QLNQLINALLRNAGQNK	GKG-----Q	37
		<u>D</u> RME		

	D	D	
VR2332	KKKNPEKPHFPLATEDDVRHHFTP	SERQLCLSSIQTAFNQAGTCTLSDS	95
LV	KKKKPEKPHFPLAARDPDRHHL	TQTERSICLOSTQTFNQAGTASLSSS	96
LDV	KKKKQPKLHFPMAGPSDLRHVMT	PNEVQMCRSSLVTLFNOGGGOCTLVDS	87

VR2332	GRISYTVFESLPTHHTVRLIRVTASPSA----	123
LV	GKVSFQVEFMLPVAHTVRLIRVTSTASOGAS	128
LDV	GGINFTVSFMLPTHATVRLINASANSSA----	115

# INTERNATIONAL SEARCH REPORT

In national Application No

PCT/NL 98/00251

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/08 C07K16/10 A61K39/12 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 04010 A (UNIV MINNESOTA) 15 February 1996  see claims; examples 12,13 ---	1,7,11, 17,19, 20,24-26
X	GB 2 289 279 A (IBERICA CYANAMID) 15 November 1995  see claims; examples 6,9-14 ---	1,7,11, 17,19, 24-26
A	WO 96 06619 A (PAUL PREM S ; MENG XIANG JIN (US); HALBUR, PATRICK (US); MOROZOV IGO) 7 March 1996 see claims; examples ---	1-27
A	WO 92 21375 A (STICHTING CENTR DIERGENEESKUND) 10 December 1992 see claims; examples ---	1,7,11, 19
-/--		

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☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

24 September 1998

Date of mailing of the international search report

01/10/1998

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International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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T	<p>J.J.M. MEULENBERG ET AL.:  "Posttranslational Processing and  Identification of a Neutralization Domain  of the GP4 Protein Encoded by ORF4 of  Lelystad Virus"  JOURNAL OF VIROLOGY,  vol. 71, no. 8, August 1997, pages  6061-6067, XP002045978  see the whole document  -----</p>	1-27

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 98/00251

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9604010 A	15-02-1996	CA 2196555 A EP 0783321 A JP 10504191 T	15-02-1996 16-07-1997 28-04-1998
GB 2289279 A	15-11-1995	ES 2078187 A AU 2409495 A DE 19517773 A DK 53595 A EP 0717108 A WO 9531550 A ES 2085837 A FR 2719845 A IT T0950367 A JP 9500544 T NL 1000365 C NL 1000365 A	01-12-1995 05-12-1995 16-11-1995 14-11-1995 19-06-1996 23-11-1995 01-06-1996 17-11-1995 13-11-1995 21-01-1997 02-10-1996 13-11-1995
WO 9606619 A	07-03-1996	CA 2198461 A EP 0776209 A	07-03-1996 04-06-1997
WO 9221375 A	10-12-1992	AT 118352 T AU 2196592 A CA 2103460 A DE 69201441 D DE 69201441 T DK 587780 T EP 0587780 A ES 2069435 T GR 3015118 T HU 67060 A JP 6508131 T MX 9202724 A US 5620691 A	15-03-1995 08-01-1993 07-12-1992 23-03-1995 01-06-1995 10-07-1995 23-03-1994 01-05-1995 31-05-1995 30-01-1995 14-09-1994 31-12-1992 15-04-1997